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Kinetic and equilibrium metal-ion-binding behaviour reflected in a metal-ion-dependent antigenic determinant in bovine prothrombin

Comparison with bovine prothrombin fragment 1

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Rabbit anti-(bovine prothrombin fragment 1) antibodies were fractionated by using fragment-1 affinity chromatography in the absence of metal ions, and showed an absolute requirement for the presence of metal ions in their interactions with bovine fragment 1 or prothrombin. These antibodies were employed to evaluate both the rate constants for a protein conformation change and the equilibrium metal-ion binding to isolated bovine fragment 1 and intact prothrombin. The close similarity of the rates obtained for the conformation change in fragment 1 and those observed in prothrombin indicated that the same process is involved in both proteins and that the non-fragment-1 region of prothrombin has essentially no effect on this process in the fragment-1 region. Equilibrium metal-ion-binding studies indicate that the details of the metal-ion-binding process in fragment 1 and prothrombin are essentially the same. We conclude that the metal-ion-binding behaviour of the fragment-1 domain of intact prothrombin is identical with that of isolated fragment 1.

The Factor X*a*-catalysed proteolytic activation of bovine prothrombin to thrombin, in a system in which the thrombin formed is not inhibited, yields three protein species: two prothrombin activation fragments, 1 and 2, and the two-chained thrombin molecule (Stenn & Blout, 1972). Gitel *et al.* (1973) demonstrated that the fragment-1 region of prothrombin is responsible for the metal-ion-dependent phospholipid-binding behaviour of the prothrombin molecule.

Previous studies from this laboratory (Marsh *et al.*, 1979*a*; Madar *et al.*, 1980) and the reports by Nelsestuen (1976) and Prendergast & Mann (1977) indicate the existence, in the absence of metal ions, of an equilibrium between two structural conformers of bovine prothrombin fragment 1. This equilibrium is illustrated by Scheme 1. Conformer A has intrinsic fluorescence properties virtually identical with those of conformer B. Conformer B is unique in that it is able to interact with Ca^{2+} in a manner that rapidly yields a conformation of fragment 1 (conformer C) that exhibits approx. 40% less-intense intrinsic fluorescence and that will interact with appropriate phospholipid surfaces. The reported energetics and comparison of the primary amino acid sequences of human and bovine prothrombin fragment 1 suggest that the interconversion of forms A and B involves the isomerization of a prolyl imino bond (Pro-22 in the bovine fragment-1 molecule). Thus the metal-ion-complexed form of fragment 1 must require a specific isomer of this amino acid, and hence of the six-residue disulphide loop in which it is contained, in order to assume a conformation capable of interacting with phospholipid surfaces. It

Abbreviations used: fragment 1, *N*-terminal portion of prothrombin released on treatment of prothrombin with thrombin; SDS, sodium dodecyl sulphate; AIC antibody, anti-(fragment 1) antibody that is independent of the presence of Ca^{2+}; ASCC antibody, anti-(fragment 1) antibodies specific for the Ca^{2+}-induced conformation of fragment 1; Gla, γ-carboxyglutamic acid.

$$A \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} B \underset{-Ca^{2+}}{\overset{+Ca^{2+}}{\rightleftharpoons}} C$$

Slow Fast

Scheme 1. *Model characterizing conformational processes involving bovine prothrombin and fragment 1*
In the absence of metal ions, two protein conformations (A and B) are in equilibrium with one another. In the presence of Ca^{2+}, conformer B is converted into conformer C. Only conformer C can interact with phospholipid.

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may further be inferred that this conformation is required for quenching of the intrinsic fluorescence of the fragment-1 molecule that is induced by the presence of metal ions.

In an effort to develop means of observing metal-ion-dependent protein conformational transitions in polypeptides lacking convenient spectroscopic properties, such as fluorescent groups, or in milieus of high complexity, such as plasma, we have raised in rabbits, and subsequently fractionated on fragment-1 affinity columns, anti-(fragment 1) antibodies. These antibodies exhibit a total dependence on the presence of metal ions, particularly Ca^{2+}, for the occurrence of the antigen–antibody reaction (Madar *et al.*, 1980). It has proved possible to utilize these antibody populations to determine the forward and reverse rate constants (k_{+1} and k_{-1} respectively) in fragment 1 for the conformational processes shown in Scheme 1. The rates obtained by using this immunokinetic approach are identical with those obtained by fluorescence-quenching methods. Thus in the presence of metal ions the time-dependent development of antigenicity in fragment 1 appears to be congruent with the conversion of fragment-1 conformer A into B.

The fragment-1 region of prothrombin has been a convenient and frequently employed model for studies of the metal-ion- and phospholipid-binding characteristics of the intact prothrombin molecule because of its convenient spectroscopic properties and because of the potential to diminish non-specific metal-ion binding. It is, however, important to examine the existence of structural constraints imposed by the remainder of the prothrombin molecule on the antigenic behaviour of the fragment-1 region (residues 1–156). Furthermore, several reports have suggested that some interactions may exist between prothrombin activation fragments and the remainder of the intact prothrombin molecule. Bloom & Mann (1978) have discussed evidence supporting the contention that conformational changes occur in the prothrombin molecule under environmental circumstances that trigger conformational changes in the fragment-1 region. A Ca^{2+} titration of the c.d. Cotton effect at 231nm, which involves side-chain chromophores, for human prothrombin yields a modified Hill coefficient, h, of 1.8 and $K_d^{app.} = 0.16$ mM. A similar titration of human fragment 1 yielded a Hill coefficient of 2.8 and $K_d^{app.} = 0.25$ mM.

Bloom & Mann (1979) also utilized c.d. to examine the question of whether indeed the various activation fragments exist in the intact prothrombin molecule as non-interacting domains. It was reported that, within probable experimental error, the prothrombin spectrum can be considered to be the independent sum of the spectra of its activation-fragment domains.

However, such observations cannot preclude subtle interactions. Transmission of effects between domains in prothrombin would more probably be apparent in folding properties of the molecule during renaturation with metal ions. Thus we have chosen to investigate folding by using modified Hill plots inasmuch as Ca^{2+} binding to fragment 1 and prothrombin is a perceptibly co-operative process.

In addition to such direct spectroscopic structural studies, quantitative metal-ion-binding studies performed in a number of laboratories suggest similarity betwen fragment 1 alone and intact prothrombin with regard to metal-ion binding. For example, Ca^{2+} binding to both proteins yields downward-curving Scatchard plots. However, in most cases subtle differences in metal-ion binding may exist. For example, Furie *et al.* (1976) examined $^{153}Gd^{3+}$ binding to bovine prothrombin and fragment 1 and obtained stoicheiometries and affinities of 1.88:1 (Gd^{3+}:prothrombin) and $K_d = 1.8\,\mu$M respectively, and 1.84:1 (Gd^{3+}:fragment 1) and $K_d = 0.16\,\mu$M respectively. Nelsestuen (1976) compared the metal-ion-induced equilibrium phospholipid binding of bovine prothrombin and fragment 1 as a function of the total Ca^{2+} concentrations. Hill plots constructed from these data yielded $h = 5$ and $K_d^{app.} = 0.096$ mM for prothrombin and $h = 3.4$ and $K_d^{app.} = 0.18$ mM for fragment 1. These data suggest that metal-ion-dependent co-operative processes involved in phospholipid binding may indeed reflect interactions between the fragment-1 domain and the remainder of the prothrombin molecule.

Recent immunochemical investigations by Lau *et al.* (1979) also suggest that the conformation of the fragment-2 region of prothrombin alone or in fragment 1–fragment 2 is different from the conformation of the same region in intact prothrombin. Furie *et al.* (1978) have reported that fragment 1 and prothrombin compete equally well with ^{125}I-labelled prothrombin for an anti-prothrombin antibody that has been fractionated in the presence of Ca^{2+} on a prothrombin-(12–44)-peptide–Sepharose column.

Thus a complex picture results from studies such as those described above. The difficulty with c.d. and direct metal-ion-binding studies lies in their fundamental lack of specificity. It is difficult to know that comparisons made involve the same region in fragment 1 and prothrombin. Equilibrium comparisons of antigenicity of fragment 1 and prothrombin compare conformational aspects of protein–metal-ion complexes and suggests their virtual identity *vis-à-vis* the antigenic site. However, the nature of this antigenic site has not been elucidated in a manner that allows conclusions to be drawn with regard to overall structural similarities.

We now present below the results of immunochemical studies designed to assess the kinetic and

equilibrium metal-ion-binding behaviour of bovine prothrombin and allow comparison of these properties with those of bovine prothrombin fragment 1.

Materials and methods

Chemicals

All reagents used were reagent grade or better. Aqueous solutions were prepared with distilled water that had been passed through ion-exchange and charcoal filters. Ultrapure $CaCl_2$, $MgCl_2$, $EuCl_3$ and $GdCl_3$ were obtained from Alfa Chemicals (Danvers, MA, U.S.A.). $MnCl_2$ was purchased from Allied Chemical Co. (Morristown, NJ, U.S.A.).

Purification of bovine prothrombin

All operations were performed at 4°C. Bovine prothrombin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) (cat. no. F4253, lot 996-3950). Approx. 40mg of this prothrombin was dissolved in 50mM-sodium citrate buffer, pH6.0, containing 1mM-di-isopropyl phosphorofluoridate and was dialysed against 50mM-sodium citrate buffer, pH6.0. The dialysed sample was applied to a DEAE-cellulose (Schleicher and Schuell) column (0.9cm x 20cm) and eluted with a linear 0–0.14M-NaCl gradient. A small wash-through peak (approx. 0.75% of applied protein) was observed, and a large prothrombin peak was eluted with the gradient. The prothrombin was concentrated with a 70%-satn.-$(NH_4)_2SO_4$ cut, and dissolved in 2ml of 50% (v/v) glycerol and stored at −20°C. The purity of the prothrombin was established by SDS/polyacrylamide-gel electrophoresis. The intrinsic fluorescence of the bovine prothrombin was quenched by Ca^{2+} in a time-dependent manner, with an equilibrium 6.1% decrease in prothrombin fluorescence in the presence of Ca^{2+}.

[^{125}I]*Iodination of bovine prothrombin with the use of chloramine-T*

The efficiency of prothrombin iodination with the use of the Bolton–Hunter reagent was only approx. 25%. Therefore iodination was done with the use of chloramine-T, in the presence of Ca^{2+}. The experiment was organized as follows. A 100μl portion (approx. 740μg) of prothrombin in glycerol was dialysed against 150ml of 0.14M-NaCl/0.5M-Tris/HCl buffer, pH7.4. After the dialysis a 10μl portion of prothrombin and 10μl of 90mM-$CaCl_2$ were mixed and incubated at 37°C for 15min, and then 10μl of $Na^{125}I$ (1mCi) was added followed by 10μl of chloramine-T (10mg/4ml of water) and 100μl of sodium metabisulphite (3mg/3.9ml of water). The reaction time was 15s. The product was separated by chromatography on a Sephadex G-25 column in 0.14M-NaCl/50mM-Tris/HCl buffer, pH7.4, containing 2mg of ovalbumin/ml. Labelling efficiency was 50%, and 98% of the label was precipitated by reaction with AIC antibody.

Preparation of antibody

The production and isolation of antiserum, described elsewhere (Madar *et al.*, 1980), involved hyperimmunization of New Zealand White rabbits with bovine prothrombin fragment 1 emulsified in Freund's complete adjuvant (Difco) over a 1-month period followed by monthly boosters of fragment 1 in incomplete adjuvant (Difco). Animals were bled 10–20 days after the booster injections. The serum was separated from the blood clot by centrifugation at 3000rev./min in a Sorvall RC-3 centrifuge and frozen at −20°C until use. Antisera were not pooled.

A fragment-1 affinity column was utilized to adsorb specifically all antibodies that bind to fragment 1 in the absence of metal ions (Madar *et al.*, 1980). Antibodies that were not retained by the column were collected in the protein wash, dialysed against 0.14M-NaCl/50mM-Tris/HCl buffer, pH7.4, and stored at 4°C until use. These antibodies, designated ASCC antibody, were used for the experiments described in the present paper.

Titre of ASCC antibody with bovine prothrombin and bovine fragment 1

Since it is important for the understanding of the results given in the present study that bovine prothrombin and fragment 1 act similarly to the

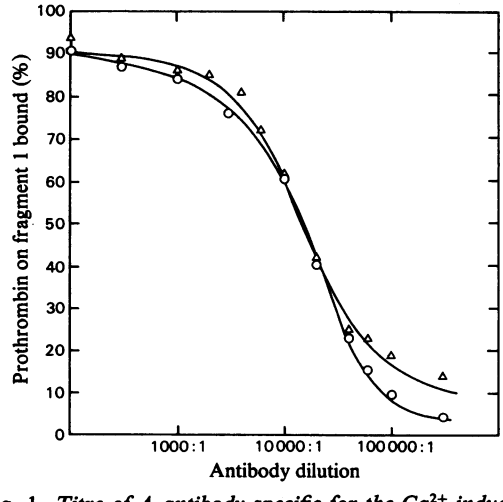

Fig. 1. *Titre of A antibody specific for the Ca^{2+}-induced conformation of fragment 1 (ASCC antibody) with bovine prothrombin* (△) *and bovine fragment 1* (○)

Experimental details are given in the text. Curves shown were obtained in the presence of 5mM-Ca^{2+}. In the absence of Ca^{2+}, less than 1% of labelled protein was bound to antibody.

antibodies previously isolated and shown to be specific for the Ca^{2+}-induced conformation of fragment 1 (ASCC antibody), the titre of ASCC antibody with both proteins was obtained in the presence and absence of Ca^{2+}. These results are shown in Fig. 1. In the absence of Ca^{2+} less than 1% of labelled protein was bound to antibody over the ASCC-antibody dilution range 1:100–1:500000. In the presence of 4mM-Ca^{2+} binding was observed, and titre curves for bovine prothrombin and fragment 1 were essentially identical. The question of damage to the Ca^{2+}-dependent antigenic sites of prothrombin by radiolabelling was addressed by observing competition of labelled and unlabelled prothrombin for the antibody. The ratio of unlabelled prothrombin to labelled prothrombin was varied from 1:10 to 10:1. A 50% inhibition of antibody binding by labelled prothrombin was obtained at a ratio of unlabelled prothrombin to labelled prothrombin of 0.9:1. This suggests that labelled and unlabelled prothrombin molecules are essentially equally antigenic.

Studies of binding of ASCC antibody to prothrombin and to fragment 1 at various metal-ion concentrations

The binding of fragment 1 to ASCC antibody in the presence of various metal ions was investigated as a function of the metal-ion concentration. Equal portions (approx. 2ng, 0.01μCi) of fragment 1 were equilibrated with appropriate dilutions of metal ions in 0.14M-NaCl/50mM-Tris/HCl buffer, pH 7.4, containing 2mg of ovalbumin (Sigma)/ml. Portions of ASCC antibody were added to each tube and allowed to incubate for 6h at room temperature (25°C). The dilution of ASCC antibody used with each metal ion was calculated as the dilution required to bind over 50% of added fragment 1. The antibody-fragment 1 was separated from free fragment 1 by addition of 50μl of a 10% (w/v) suspension of IgG-sorb (Enzyme Center, Boston, MA, U.S.A.) followed by sedimentation of the bacteria after 10min by centrifugation at 3000rev./min for 1min in an Eppendorf model 3412 microcentrifuge. The pellets were washed once with buffer before counting of radioactivity on a Beckman Gamma 4000 scintillation system. Metal-ion concentrations investigated were between 0.001 to 10mM. Hill plots were constructed from the binding data by calculation of the ratio of the concentration of metal-ion-binding sites filled to the concentration of sites vacant. The sites filled at any metal-ion concentration were calculated by assuming that in the presence of excess of metal ion all sites would be filled and that at lower metal-ion concentrations the sites filled would be proportional to the amount of radioactivity precipitated. Hill plots (Van Holde, 1971) of log[(sites occupied)/(sites vacant)] were generated from the antibody-binding data as follows:

$$\frac{\theta}{1-\theta}=\left(\frac{CPM_{obs.}-NSB}{CPM_{max.}-NSB}\right)\Big/(1-\text{numerator})$$

where $CPM_{obs.}$ is the observed radioactivity (c.p.m.) precipitated at a given metal-ion concentration, $CPM_{max.}$ is the maximum precipitable radioactivity (c.p.m.) and NSB is non-specific background radioactivity (c.p.m.).

Kinetic studies of the metal-ion-induced prothrombin conformational change [forward reaction ($A \rightarrow B$, Scheme 1)]

Antibody from the wash-through of the affinity column (ASCC antibody) was pre-equilibrated with an equal volume of 20% (w/v) suspension of IgG-sorb. Radiolabelled fragment 1 (or prothrombin) was added to an equal volume of a buffer solution containing 9mM-$CaCl_2$ and allowed to react for various times. Then 50μl of the IgG-sorb–antibody suspension was added, allowed to react for 10min and sedimented by centrifugation. The pellet was re-suspended in 0.2ml of buffer, re-sedimented and its radioactivity counted. The kinetics were examined with reagents and materials equilibrated at the temperatures specified in a Forma Scientific 12L water bath. The times examined ranged from 10min to 2h. The t_∞ value was obtained by incubation for 16h. Rate constants were obtained from the slope of a plot of $\ln[(t-t_0)/(t-t_\infty)]$ versus reaction time, where t is the percentage of fragment 1 bound at any time and the reaction time is taken as the total time elapsed after addition of fragment 1 before centrifugation. The t_0 was taken as the non-specific background value.

Results

Kinetic behaviour

The kinetic behaviour of bovine prothrombin after addition of 2mM-$CaCl_2$ is illustrated in Fig. 2. These results are consistent with Scheme 1 and results previously obtained for bovine prothrombin fragment 1 by both immunological and fluorescence methods, since immediately on addition of Ca^{2+} approx. 30% of prothrombin binds to antibody specific for conformer C (Scheme 1). Those prothrombin molecules were presumably in conformation B when Ca^{2+} was added and very rapidly interacted with Ca^{2+} to yield state C. The subsequent slow increase in the percentage of prothrombin bound reflects the slow conversion of conformer A into conformer B, which is then rapidly converted into conformer C in the presence of Ca^{2+}. The observed kinetics fit the expression for a first-order process (Fig. 2*b*). Kinetic data acquired at 4°C and 10°C

and with Ca^{2+}, Mg^{2+} and Mn^{2+} as triggers of the conformation change are collected in Table 1.

The rate constant of the conformation change determined with Ca^{2+} and prothrombin at 4°C is (within a probable error of 10%) identical with that obtained for fragment 1 under the same conditions. The fact that the rates of the prothrombin and fragment-1 conformation changes are essentially the same at 10°C indicates that the energy of activation of the process that is observed is the same for prothrombin and fragment 1 and is of the order of 84 kJ/mol. A similar comparison between the rates of prothrombin and fragment 1 conformation changes can be made in the presence of Mg^{2+}. Rate constants of $0.015\,min^{-1}$ and $0.017\,min^{-1}$ were obtained for prothrombin and fragment 1 respectively. These values, obtained at 4°C, are quite close to those obtained for these two proteins when Ca^{2+} is employed as the trigger for the A→B conversion. Similar results were obtained at two triggering concentrations of Mn^{2+}.

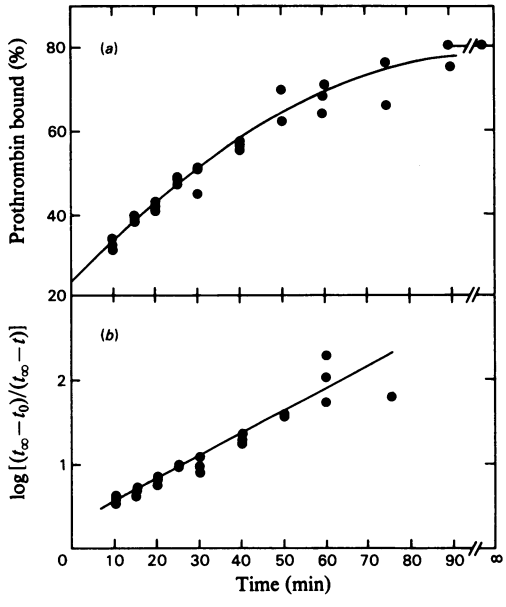

Fig. 2. *Development of antigenicity of bovine prothrombin as a function of time after addition of 2 mM-Ca^{2+}*
Experimental details are given in the text. (*a*) Percentage of prothrombin bound as a function of time after addition of Ca^{2+} at 4°C. (*b*) First-order plot of data illustrated in (*a*). The slope of the linear least-squares line drawn is $0.023\,min^{-1}$.

Equilibrium behaviour

Although the conformation change (A→B) precedes the appropriate folding of the polypeptide chain in the presence of Ca^{2+} to yield a phospholipid-binding conformation (state C), the rate of the conformational process yields little direct insight into the folding process itself. Hence metal-ion titrations were performed with the antibodies specific for the Ca^{2+}–fragment 1 or Ca^{2+}–prothrombin complex (state C) in order to assay the degree of folding. Modified Hill plots were constructed from the saturation curve obtained. The equilibrium dose–response behaviour of prothrombin to the presence of Ca^{2+} and the resulting Hill plots are illustrated in Fig. 3. The results obtained from a series of such Hill plots involving bovine prothrombin and bovine fragment 1 and a variety of metal ions are collected in Table 2.

Consistent with the results of a variety of spectroscopic techniques, the Hill coefficient, h, obtained for Ca^{2+} binding to prothrombin and fragment 1 is approx. 3.0, with an apparent dissociation constant, $K_{eq.}^{app.}$, of the metal ion–protein complex of approx. 0.2×10^{-3} M. Mg^{2+} binding to prothrombin yields a Hill coefficient of 1.0 and a slightly lower $K_{eq.}^{app.}$ value of 0.09×10^{-3} M. Mg^{2+} binding to fragment 1 yields a Hill coefficient of 1.3 and a $K_{eq.}^{app.}$ value of 0.3×10^{-3} M. Mn^{2+} binding to fragment 1 yields 0.72 and 0.15×10^{-3} M for h and

Table 1. *Immunologically determined bovine prothrombin isomerization rate constants (A→B, Scheme 1): comparison with bovine prothrombin fragment-1 rates*
For experimental details see the text.

Protein	Metal ion	Temperature (°C)	k_{+1} (min^{-1})
Prothrombin	Ca^{2+}	4	0.023
Fragment 1	Ca^{2+}	4	0.019
Prothrombin	Ca^{2+}	10	0.036
Fragment	Ca^{2+}	10	0.034
Prothrombin	Mg^{2+}	4	0.015
Fragment 1	Mg^{2+}	4	0.017
Prothrombin	Mn^{2+} (0.1 mM)	4	0.019
Prothrombin	Mn^{2+} (1.0 mM)	4	0.016

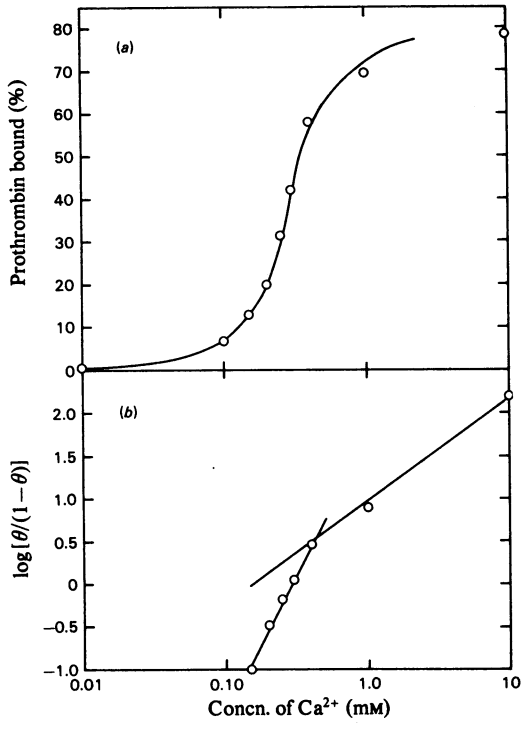

Fig. 3. *Development of prothrombin antigenicity towards ASCC antibody as a function of the Ca^{2+} concentration*

Experimental details are given in the text. (*a*) Percentage of prothrombin bound to antibody as a function of Ca^{2+} concentration. (*b*) Hill plot obtained from the data represented in (*a*) as described in the text. The maximal slope at $\log[\theta/(1-\theta)] = 0$, *h*, is 3.2 and the intercept at this point is 0.3×10^{-3} M.

Table 2. *Immunologically determined Hill-plot parameters characterizing the interaction of bovine prothrombin and bovine prothrombin fragment 1 with various metal ions*

For experimental details see the text.

Protein	Metal ion	*h**	$K_{eq.}^{app.}$‡ (M)
Prothrombin	Ca^{2+}	3.2 ± 0.1†	0.3×10^{-3}
Fragment 1	Ca^{2+}	3.2	0.18×10^{-3}
Prothrombin	Mg^{2+}	1.0	0.09×10^{-3}
Fragment 1	Mg^{2+}	1.3	0.3×10
Fragment 1	Mn^{2+}	0.72	0.15×10^{-3}
Fragment 1	Gd^{3+}	2.9	0.8×10^{-6}

* Hill-plot slope.

† Extrapolation of line of slope = 1.1 at high site occupancy yields an intercept at $\log[\theta/(1-\theta)] = 0$ of 0.12×10^{-3} M.

‡ Intercept of Hill plot at $\log[\theta/(1-\theta)] = 0$.

$K_{eq.}^{app.}$ respectively. Gd^{3+} binding yields 2.9 for *h* and 0.8×10^{-6} M for $K_{eq.}^{app.}$.

Discussion

As summarized in the introduction, comparison between isolated bovine prothrombin fragment 1 and the same region in intact prothrombin has achieved limited refinement owing to the scarcity of spectroscopic and functional properties of fragment 1 that are not obscured by the additional size and complexity of prothrombin. Thus far, the principal means of comparison have involved: demonstration of c.d.-related changes in prothrombin between its variously defined regions, suggesting that they act independently; demonstration that both fragment 1 and prothrombin interact with phospholipid in the presence of Ca^{2+}; demonstration of co-operative Ca^{2+} binding by both species; demonstration of a Ca^{2+}-induced decrease in prothrombin intrinsic fluorescence that is roughly, on a mass basis, equivalent to that which occurs in isolated fragment 1 on addition of metal ions. The results of the present immunological study add considerable support to this circumstantial case, and indicate that, from a kinetic and equilibrium protein-folding point of view, isolated fragment 1 and the corresponding region of prothrombin exhibit essentially the same behaviour.

The antigenicity of fragment 1 and prothrombin towards ASCC antibody are virtually identical in the presence of Ca^{2+}. Furthermore, neither protein reacts with the ASCC antibody in the absence of Ca^{2+}. In addition, the utilization of an immunological assay system has allowed the experiments reported in the present paper to be performed at concentrations considerably lower than those at which protein–protein associative processes are likely to occur.

Independent of specific assumptions about the molecular events inherent in the conformational relaxation involved in the metal-ion-dependent evolution of an antigenic site on prothrombin or fragment 1, it is reasonable to conclude from the kinetic results presented above that the same process is rate-limiting in folding of prothrombin and fragment 1. This kinetic process involves the presence of metal ions, but is not explicitly dependent on the specific metal ion involved or its concentration, as long as the concentration is sufficiently high. These results are consistent with the model presented in Scheme 1. Since the rate-limiting process is the conversion of form A into form B, any method that assays for form C must yield similar results. Since the intrinsic fluorescence of state C of fragment 1 is significantly quenched relative to states A and B, fluorescence-quenching measurements yield identical kinetic parameters (Nelsestuen, 1976; Prendergast & Mann, 1977;

Marsh *et al.*, 1979*b*). Similarly, since phospholipid binding requires the existence of state C, the rate of phospholipid binding must reflect this A→B kinetic process. Indeed, Nelsestuen (1976) has evaluated the rates of phospholipid binding by bovine prothrombin and fragment 1 after addition of Ca^{2+} and reports essentially identical rates. Thus the isomerization process is linked to fluorescence quenching, to phospholipid binding and to development of a metal-ion-dependent antigenic site in fragment 1 and prothrombin. In terms of a structural hypothesis we may conclude that in bovine prothrombin and in bovine fragment 1 the conformation about Pro-22 and hence the geometry of the Cys-18–Cys-23 disulphide loop is crucial to appropriate metal ion–protein interactions that lead to a phospholipid-binding form of the protein.

The interaction of a wide variety of metal ions with form B leads rapidly to a form C (which may be unique for each particular metal ion) in which fluorescence is quenched and which attains to a greater or smaller extent the antigenic structure recognized by ASCC antibody. Since the thermodynamic free energies of forms A and B are quite similar, although they are separated by a sizeable kinetic barrier, we may speculate that forms A and B differ in only a very limited way. This conclusion is required by the identical fluorescence of forms A and B and the relative absence of structure in either form. Thus we may further speculate that the primary structural constraint characterizing states A and B, at neutral pH in the absence of metal ions, are electrostatic repulsive forces involving the ten Gla residues' ionized carboxylic acid side chains. The fact, then, that most metal ions are able to yield a generally similar fragment 1 or prothrombin structure indicates that in the presence of metal ions these repulsive forces are largely relieved, allowing the main chain to fold to a new, more stable, conformation. It would further seem logical that statistically the most satisfactory initial metal-ion-binding site would involve a region of organized secondary structure, i.e. the region of the Cys-18–Cys-23 loop and the associated Gla residues.

Elsewhere (Marsh *et al.*, 1979*b*; Madar *et al.*, 1980) we have developed the argument that the final state C yielded by fragment 1 or prothrombin in the presence of a variety of metal ions depends to a considerable extent on the metal ion involved and that, owing to the size, charge and co-ordination geometry of Ca^{2+}, these ions are uniquely able to induce in fragment 1 and prothrombin a conformation capable of interacting with phospholipid. One potential reflection of this unique capacity of Ca^{2+} is apparent in the data collected in Table 2. A large and positive Hill coefficient is observed for Ca^{2+} binding to fragment 1 and prothrombin. Thus the Ca^{2+}-binding sites interact via protein backbone-folding processes. The identity of Hill coefficients obtained for Ca^{2+} binding to fragment 1 and to prothrombin suggests that this folding process induced by Ca^{2+} occurs independently of the non-fragment-1 portion of prothrombin. In contrast with the behaviour of Ca^{2+} with these proteins, Mg^{2+} appears to bind without co-operativity. Furthermore, non-co-operative or perhaps even negatively co-operative binding of Mn^{2+} to fragment 1 is observed.

The Mn^{2+}-dependent development of antigenicity reveals an apparent dissociation constant of 0.15×10^{-3} M. Over the Mn^{2+} concentration range investigated, the percentage bound varied from 5 to 70, indicating that the reported values reflect the behaviour of the bulk of the fragment-1 molecules. Nelsestuen *et al.* (1976) have reported that the rate of the slow fluorescence decrease in bovine prothrombin fragment 1 (the A→B conformational change) is dependent on the concentration of Mn^{2+} employed to trigger the transition. The time at 80% reaction was observed to decrease by approximately 10-fold over the Mn^{2+} concentration range 0.1–1.0 mM. As assessed by development of antigenicity in prothrombin, essentially no change in the rate of the slow conformational transition is apparent at Mn^{2+} concentrations of 0.1 and 1.0 mM (Table 1). Thus we suggest that the apparent increasing rate of fluorescence quenching observed in the presence of increasing Mn^{2+} concentration may be explained by a model in which it is assumed that Mn^{2+} bound to sites on fragment 1 is able to paramagnetically quench the tryptophan excited state. The e.s.r. binding studies by Bajaj *et al.* (1976) suggest the existence of at least one relatively high-affinity Mn^{2+}-binding site in prothrombin and fragment 1 (K_d approx. 12 μM) and lower-affinity sites in the 0.1 mM range. Our previously reported study of immunologically determined equilibrium binding of Mn^{2+} to bovine prothrombin fragment 1 indicates that the development of fragment-1 antigenicity involves an average dissociation constant of 0.15×10^{-3} M (Madar *et al.*, 1980). The results obtained by Nelsestuen *et al.* (1976) similarly implicate binding processes in the 0.2 mM-Mn^{2+} concentration range. Thus we may tentatively conclude that the high-affinity Mn^{2+}-binding sites may not be directly reflected in protein folding, assessed immunologically, or in fluorescence-quenching processes involving the fragment-1 region. Employing a different antibody preparation, Furie & Furie (1979) have observed a pronounced increase in prothrombin–antibody interactions as a function of Mn^{2+} concentration with an estimated half-maximal effect at 20 μM, suggesting that their antibody population is possibly sensitive to structural alterations in the prothrombin molecule associated with binding of Mn^{2+} to high-affinity sites on prothrombin.

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